



# NO Microsensors

*ISO-NOPF100, ISO-NOPF200, ISO-NOPF-200-L10, ISO-NOP70L,  
ISO-NOP3005, ISO-NOP3020 ISO-NOP30-L, ISO-NOP007, ISO-NOPNM*

## **INSTRUCTION MANUAL**

Serial No. \_\_\_\_\_

071009

[www.wpiinc.com](http://www.wpiinc.com)



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## ABOUT THIS MANUAL

The following symbols are used in this guide:



This symbol indicates a **CAUTION**. Cautions warn against actions that can cause damage to equipment. Please read these carefully.



This symbol indicates a **WARNING**. Warnings alert you to actions that can cause personal injury or pose a physical threat. Please read these carefully.

NOTES and TIPS contain helpful information.

## INTRODUCTION

The **NO Microsensors** are available in a variety of tip diameters and lengths for a range of applications. These microsensors incorporate WPI's proprietary combination electrode technology whereby the composite graphite nitric oxide sensing element and separate reference electrode are encased within a single sensor design.

- Used for in vivo applications, the **ISO-NOPF** sensors are available in 100 $\mu$ m (WPI #**ISO-NOPF100**) and 200 $\mu$ m (WPI #**ISO-NOPF200**) diameters. They were created to be flexible and are the most durable of all the WPI's nitric oxide sensors. The **ISO-NOPF** is also available with a hypodermic sheath (WPI #**ISO-NOPF100H** or **ISO-NOPF200H**). These sensors are sold in 1-5mm lengths of whole 1mm increments (1mm, 2mm, 3mm, 4mm, 5mm).
- For use with a tissue bath or in monolayer-style cell culture studies, the **ISO-NOPF200** is available in an L-shape (**#ISO-NOPF200-L10**), and it is 10mm long. The L-shaped version is also available in 70 $\mu$ m (WPI #**ISO-NOP-70-L**) or 30mm (WPI #**ISO-NOP30-L**) diameters, both 3mm long.
- When working with microvessels, three options are available. The **ISO-NOP007** is 7 $\mu$ m in diameter and 2mm long. The **ISO-NOP3005** is 30 $\mu$ m in diameter and 0.5mm long. And, the **ISO-NOP3020** is 30 $\mu$ m in diameter and 2mm long.
- For single cell applications, the conical tip of the **ISO-NOPNM** is only 100 $\mu$ m and the sensor tip is 150 $\mu$ m long.

**Fig. 1** (on the next page) shows a labeled drawing of the carbon fiber microsensors.

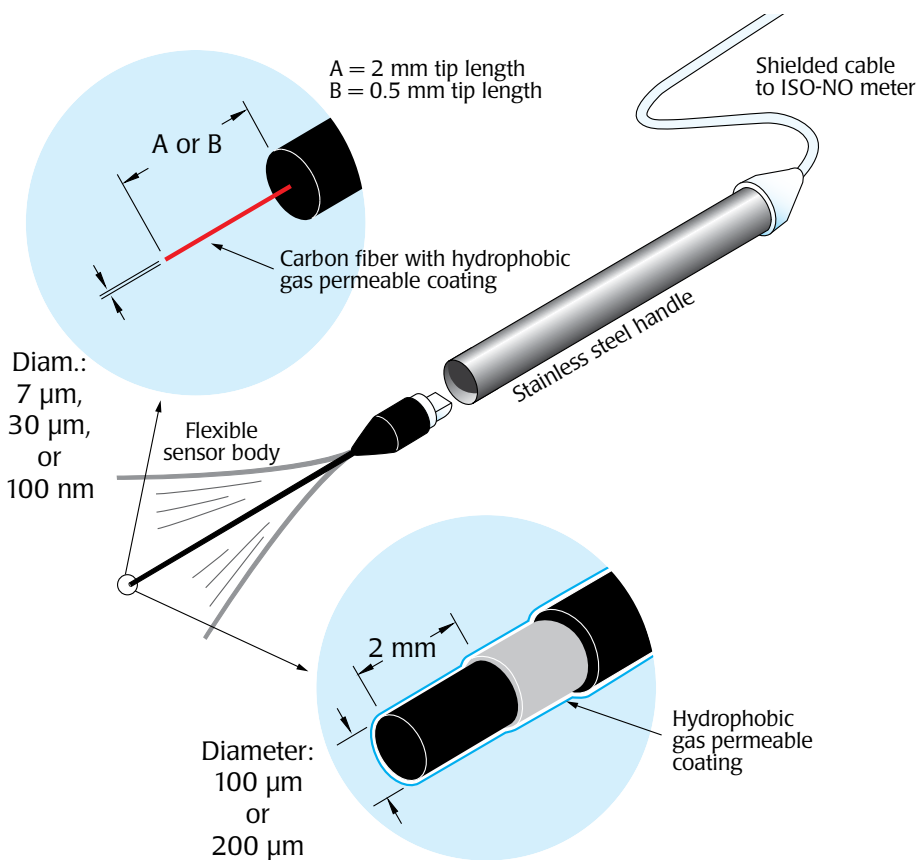


Fig. 1—ISO-NOPF microsensor with cable

## Notes and Warnings

The NO carbon fiber microsensors are robust, but not indestructible. Exercise caution when handling the NO sensor to avoid actions that could damage the tip. Do not bring the tip into contact with hard surfaces like stir bars. See "Unpacking" on page 3.



**CAUTION:** DO NOT EXPOSE SENSOR TO ORGANIC SOLVENTS.



**CAUTION:** Carefully read the "Probe Unpacking" instructions (found in the sealed sensor case) before handling the sensor.

## INSTRUMENT DESCRIPTION

### Parts List

After unpacking, verify that there is no visible damage to the sensor. Verify that all items are included:

- (2 or 3) NO microsensors (quantity depends on the type of sensor)
- (2 or 3) Sensor Performance Evaluations (each sensor is tested individually at WPI)
- (1) Instruction Manual

### Unpacking

Upon receipt of these sensors, make a thorough inspection of the contents and check for possible damage. Missing cartons or obvious damage to cartons should be noted on the delivery receipt before signing. Concealed damage should be reported at once to the carrier and an inspection requested. Please read the section entitled "Claims and Returns" on page 23 of this manual. Please contact WPI Customer Service if any parts are missing at 941.371.1003 or [customerservice@wpiinc.com](mailto:customerservice@wpiinc.com).

The microsensors are shipped in a sealed, rigid plastic, hinged box with foam padding to protect them from damage during shipment.

To open the package, carefully cut the seals on either side of the sensor box. The sensors are packaged in foam holders so that their tips are physically isolated from contact with the container. To remove a microsensor from the package, use the thumb and index finger of one hand to separate the slit in the foam which contains the sensor and use the thumb and index finger of the other hand to grasp the sensor at its midsection and gently remove it from the container.

KEEP THE SENSOR STORAGE BOX and the documentation in a safe place. The test date and serial number of each sensor is printed on the bottom of its box. Use of the sensor should begin within 30 days of receipt.

**Returns:** Do not return any goods to WPI without obtaining prior approval (RMA # required) and instructions from WPI's Returns Department. Goods returned (unauthorized) by collect freight may be refused. If a return shipment is necessary, use the original container, if possible. If the original container is not available, use a suitable substitute that is rigid and of adequate size. For further details, please read the section entitled "Claims and Returns" on page 23 of this manual.

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# OPERATING INSTRUCTIONS

## Environmental Influences

There are two environmental parameters to which NO sensors are quite sensitive: temperature and electrical interference.

### Temperature

The background current (and to a lesser degree) the selectivity of the NO sensor is affected by temperature. This is due to the effects of temperature on the partial pressure of dissolved NO gas in liquid samples, on the permeability of the coatings and on the conductivities of various sensor components. It is recommended that a calibration procedure be performed at the same temperature as the experiment and that temperature be held constant during NO measurement.

### Electrical Interference

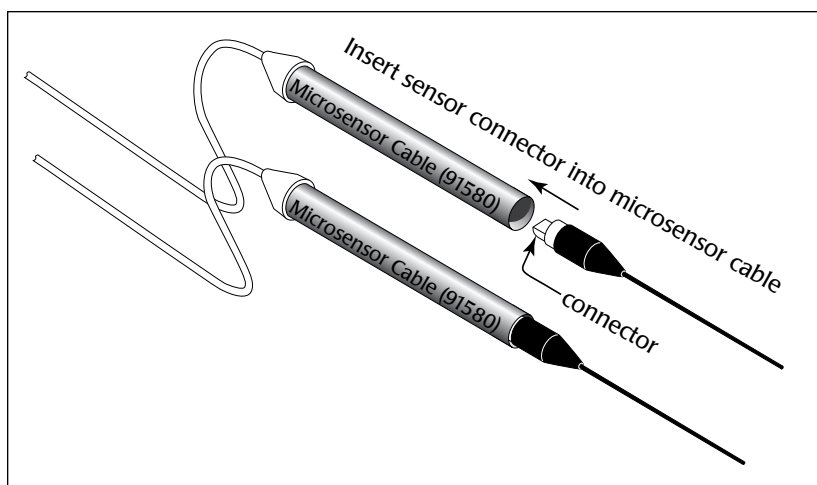
External, electrical noise sources (like magnetic stirrers, fluorescent lights, MRI machines, electric motors, computers, pumps and other electrical instruments) may couple into the sensor signal path electromagnetically and impose undesirable signals in the output record. The magnitude of this external noise depends on the environment of the laboratory. If the interference introduced by the electrical signals in the environment is large, identify the noise source and remove it. It is also important to ground and shield the system properly.

**TIP:** Refer to your free radical analyzer manual for proper grounding and shielding techniques. (In the TBR4100 or Apollo1000 manuals, see “Grounding and Noise Concerns” in the Operating Instructions section.)

## Attaching the Sensor to the Microsensor Handle

Once removed from the package, plug the microsensor into a microsensor cable (WPI #91580) connected to the free radical analyzer (Fig. 2).\* **Be very careful that the sensor tip does not come into contact with anything which could damage it.** The sensor should plug in easily. If you encounter resistance, it is probably due to misalignment of the sensor plug with the socket connector inside the microsensor cable. Simply realign the sensor by gently rotating it until it snaps into place.

**\*NOTE:** Current WPI free radical analyzers include the TBR4100, TBR1025 and Apollo1000. Apollo4000 is the original 4-channel WPI free radical analyzer.



**Fig. 2**—NO microsensors may be changed or replaced quickly and easily

## Polarizing the Sensor

When a non-polarized microsensor is initially connected to a free radical analyzer, it may display a high (sometimes off-scale) background current. The polarization voltage applied by the instrument causes a reduction of the background current to a stabilized baseline value over time. Set the poise voltage to 865mV. (For the TBR4100/1025, set the **Probe Select** dial to **NO**.) The amount of time required to reach a stable baseline current varies for each sensor. New sensors typically take longer, on the order of several hours.

The Performance Evaluation included with your sensor shows the baseline current and the sensitivity of your sensor when it was quality tested at WPI. (In addition, it shows the polarization time of your sensor in the WPI labs.) The baseline value attainable in your lab may be slightly higher or lower, depending on the temperature\* and composition of the test media. For initial performance verification of an **ISO-NOPF** in your lab, WPI recommends using the  $\text{CuCl}_2$  calibration method (Method 2) described on page 8. Once a stable baseline current is achieved (usually between 500-8000pA), the microsensor is ready for use.

**\*NOTE:** The background current of the sensor will usually increase with increasing temperature of the experiment. Although the sensitivity of the sensor does not change significantly within the range 20-37°C, it is recommended that the calibration procedure be performed at the same temperature as the experiment.

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## Calibrating the Sensor

Accurate measurements of NO require an accurate calibration. Three calibration methods are described in this section.

- Method 1 is based on the decomposition of the S-nitrosothiol NO-donor (SNAP) using CuCl. The NO liberated from SNAP is used to calibrate the sensor.
- Method 2 is similar to Method 1 using CuCl<sub>2</sub> as a catalyst. This convenient method is the one used in WPI laboratories. Experimentally it has been shown that CuCl<sub>2</sub> is less efficient as a catalyst in the conversion of SNAP to NO. (The conversion ratio is reduced to approximately 60%.) However, it is technically easier to accomplish than Method 1, and it provides reliable data.
- Method 3 involves preparing aqueous solutions of NO from saturated NO solutions prepared with NO gas.



**WARNING: THIS METHOD USES NO GAS WHICH CAN BE FATAL IF IT IS MISHANDLED.**

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## Understanding SNAP

SNAP is a stable NO-containing compound that can be used for quantitative generation of NO in solution. SNAP decomposes to NO and a disulfide byproduct when dissolved in water. However, the rate of decomposition is very slow. The kinetics of decomposition for this reagent is a function of several parameters including pH, presence of a catalyst, temperature and light.

In the procedures described here, SNAP is used in combination with a catalyst, cuprous (I) chloride (CuCl) or cupric (II) chloride (CuCl<sub>2</sub>), to generate a known quantity of NO in solution. Note that this protocol does not investigate the effects of all parameters involved in SNAP decomposition, nor does it propose a model by which NO is decomposed. The presented procedures provide an empirical estimation of the amount of generated NO based on the molarity of a standard (stock) solution of SNAP under a controlled set of parameters.

**NOTE:** Remember that most NO probes are sensitive to temperature changes. It is therefore recommended that the calibration of a NO sensor is performed at the experimental temperature.

### Method 1: SNAP using CuCl

This method of calibration results in the 100% conversion of SNAP to NO. The amount of NO produced, therefore, is based on the final concentration of SNAP.

**NOTE:** The described calibration procedure requires the use of cuprous chloride, CuCl, where CuCl is the active catalyst for the conversion of SNAP to NO. The calibration curve assumes only the presence of CuCl and hence a 100% conversion

efficiency of SNAP to NO. However, in the presence of oxygen CuCl is readily oxidized to CuCl<sub>2</sub>. This will happen naturally if the compound is exposed to air and/or there is inadequate storage of CuCl. The oxidation product CuCl<sub>2</sub> is much less efficient at catalyzing the conversion of SNAP to NO, and this would appear during calibration as an apparent low sensitivity of the electrode to NO. Since CuCl is readily oxidized to CuCl<sub>2</sub> special precautions must be taken to keep it in its reduced state prior to any calibration. It is recommended that CuCl be stored under inert conditions, and (if used in solution) the solution must be degassed with inert gas and absent of all oxygen.

**NOTE:** If your laboratory is not adequately equipped to satisfy the conditions for storage and use of CuCl, use the CuCl<sub>2</sub> method (Method 2) on page 8.

### ***Preparing Solutions***

- **Solution #1** (Saturated solution of CuCl): Add 150mg CuCl to 500mL distilled deoxygenated water. The saturated CuCl solution will have a concentration of approximately 2.4mM at room temperature and should be kept in the dark prior to use.

**NOTE:** The distilled water can be deoxygenated by purging with pure nitrogen or argon gas for 15 minutes.

- **Solution #2** (Standard SNAP solution): Add 5.0mg EDTA to 250mL of water and deoxygenate the solution. Then add 5.6mg of SNAP and dissolve it completely.

**TIP:** For complete instructions on making standard 100mM SNAP and calculating the molarity of SNAP solution, see Appendix A, page 16.

### ***Calibration Procedure***

1. Within a nitrogen or argon environment, place 10.0mL of Solution #1 (CuCl) in a 20mL vial.
2. Drop a small stirring bar into the solution, and place the vial on a magnetic stirring plate.
3. Immerse an NO probe into this solution. While stirring allow the sensor to polarize until the background current stabilizes. The appropriate time for stabilization and expected baseline current values depend on the model of the sensor. (See "Specifications" on page 15.)
4. As soon as the background current stabilizes, begin recording the current output.
5. Sequentially inject five aliquots containing 2μL, 4μL, 8μL, 16μL and 32μL of Solution #2 (SNAP standard solution) into the vial containing Solution #1 (CuCl). Immediately following the first addition of SNAP into Solution#1, the current (pA) output will increase rapidly. Within a few seconds the response will reach a plateau. Inject the second aliquot (4μL) as soon as the first signal reaches a plateau. Add the third aliquot (8μL) as the second signal reaches its plateau. If aliquots are not added promptly when reaching the previous plateau, the signal will slowly decline because generated NO is quickly oxidized to nitrite and

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nitrate which will not be detected by the probe. Add the fourth and fifth aliquots in the same manner.

6. Construct a calibration curve by plotting the signal output (pA) against the concentration (nM) of SNAP. (See "Appendix B: Constructing a Calibration Curve" on page 18.)

## Method 2: SNAP using CuCl<sub>2</sub>

### Preparing Solutions

- **Solution #1**(CuCl<sub>2</sub> solution): 250mL 0.1 M CuCl<sub>2</sub> in distilled water
- **Solution #2** (Standard SNAP solution): Add 5.0mg EDTA to 250mL of water. Then, add 5.6mg of SNAP and dissolve it completely.

**TIP:** For complete instructions on making standard 100mM SNAP and calculating the molarity of SNAP solution, see Appendix A, page 16.

### Calibration Procedure

1. Place 20.0mL of Solution #1(CuCl<sub>2</sub>) in a 20mL vial.
2. Drop a small stirring bar into the solution, and place the vial on a magnetic stirring plate.
3. Immerse an NO probe into this solution. While stirring allow the background current to stabilize. The appropriate time for stabilization depends on the model of the sensor. (See "Specifications" on page 15.)
4. As soon as the background current stabilizes, begin recording the current output.
5. Sequentially inject five aliquots containing 2μL, 4μL, 8μL, 16μL and 32μL of the SNAP standard solution (Solution #2) into the vial containing Solution #1 (CuCl<sub>2</sub>). Immediately following the first addition of SNAP into Solution#1, the current (pA) output will increase rapidly. Within a few seconds the response will reach a plateau. Inject the second aliquot (4μL) as soon as the first signal reaches a plateau. Add the third aliquot (8μL) as the second signal reaches its plateau. If aliquots are not added promptly when reaching the previous plateau, the signal will slowly decline because generated NO is quickly oxidized to nitrite and nitrate which will not be detected by the probe. Add the fourth and fifth aliquots in the same manner.

**TIP:** You can adjust the volume of injected aliquots according to the concentration of SNAP stock solution. Decrease the volume of aliquot if the electrode is very sensitive or increase the volume of aliquot if the electrode is less sensitive.

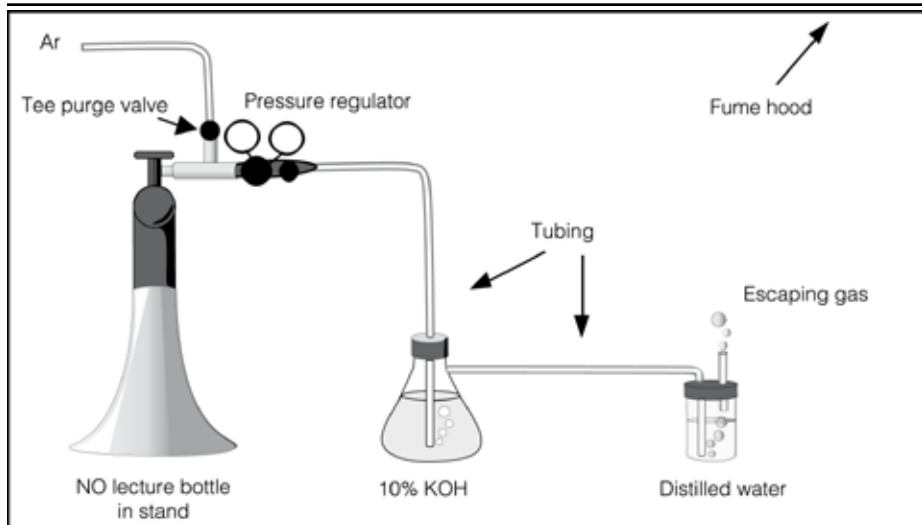
6. Construct a calibration curve by plotting the signal output (pA) against the concentration (nM) of SNAP. (See "Appendix B: Constructing a Calibration Curve" on page 18.)

### Method 3: NO Standards Prepared with NO Gas

This method can be used with all NO sensors and has the advantage of allowing you to calibrate NO sensors in the same environment in which the experimental measurements will be made. However, it has the disadvantages of added cost, inconvenience, and greater hazard to the user. All of these factors must be taken into consideration.



**WARNING: NITRIC OXIDE MUST BE HANDLED ONLY IN A WELL-VENTILATED AREA, TYPICALLY A LABORATORY FUME HOOD WITH FORCED VENTILATION. THE U.S. OCCUPATIONAL SAFETY AND HEALTH ADMINISTRATION HAS SET A TIME-WEIGHTED AVERAGE MAXIMUM NO VALUE AS 25PPM. THAT IS TO SAY, 25PPM IS CITED AS THE MAXIMUM CONCENTRATION TO WHICH WORKERS MAY BE CONTINUALLY EXPOSED. BRIEF INHALATION OF CONCENTRATIONS AS LOW AS 200PPM COULD PRODUCE DELAYED PULMONARY EDEMA WHICH MAY BE FATAL AFTER AN ASYMPTOMATIC PERIOD OF UP TO 48 HOURS AFTER THE INITIAL EXPOSURE. IT IS THEREFORE CRITICAL THAT THE PERSONNEL HANDLING THE GAS BE THOROUGHLY FAMILIAR WITH THE MATERIAL SAFETY DATA SHEET (MSDS) AND PROPER HANDLING PROCEDURES. THE PRECAUTIONS RECOMMENDED BY THE GAS MANUFACTURER MUST BE FOLLOWED.**



**Fig. 3**—Setup for preparing a saturated NO aqueous solution must be in a fume hood with forced ventilation. Nitric oxide is highly toxic, and it escapes into the atmosphere during preparation of the standards. NO Lecture bottle (14.2L, 98.5%) can be obtained from Aldrich.

1. **Be certain the fume hood is functioning. Inhalation of NO gas is potentially fatal. See the WARNING above.**

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2. Make sure that all fittings and connections are secure. The tubing to be used should not be permeable to NO. We recommend Tygon® tubing if a polymer tubing is to be used. This is permeable to NO, but it has the best performance compared to other polymer tubing of which we are currently aware. Ideally glass tubing should be used. If Tygon® tubing is used, note that prolonged exposure to NO affects its properties. Therefore, it is recommended that the tubing be inspected frequently and that it be replaced when it appears to be brittle. The pressure regulator and tee purge adaptor should be stainless steel since nitric oxide is corrosive.
  3. Prepare 100mL of a 10% (by weight) KOH solution and place it in the sidearm flask (**Fig. 3**). The flask should be sealed with a stopper through which the tubing passes by means of a Luer fitting to a syringe needle which extends almost to the bottom of the flask. Tubing is used to connect the side arm of the flask to the vial containing the water to be equilibrated with NO. The KOH solution is used to remove other nitrogen oxides from the NO gas.
  4. Place 20mL of distilled (preferably deionized) water in a small glass vial. Seal the vial with a stopper and insert (through the stopper) a long syringe needle which extends almost to the base of the vial. Connect this syringe needle to the tubing from the KOH flask, as illustrated. Insert an additional shorter syringe needle which **should not extend into the solution**. This acts as a pressure relief during purging.
  5. Place the distilled water vial in an ice-water bath. Reducing the temperature increases the solubility of NO in solution. Thus, when the solution is used at room temperature, you will be assured of a saturated NO solution.
  6. Purge the system with argon (or nitrogen) gas for a period of 30 minutes at a moderate flow rate such that the pressure is maintained at a safe level (1-2PSI). When purging, it should be observed that gas is indeed bubbling through the KOH solution, as well as the distilled water. After 30 minutes turn off the argon source and switch the tee purge valve to the correct position for purging with NO from the lecture bottle.
  7. If you are using a pure source, purge the system with NO for 5-10 minutes. (If the source is not pure, it will take longer.) Again, verify that gas is bubbling in the solution.



**WARNING: NO IS NOW ESCAPING FROM THE PRESSURE RELIEF NEEDLE IN THE STOPPER OF THE DISTILLED WATER VIAL. IT IS IMPERATIVE THAT THE FUME HOOD BE RUNNING AT MAXIMUM CAPACITY WITH THE FRONT PANEL CLOSED (IN THE DOWN POSITION).**

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8. After the time in step 7 has elapsed, turn off the NO source.
9. Immediately remove the two needles from the distilled water vial.

10. Set the tee purge valve for purging with argon (or nitrogen) gas, and turn on the argon source. Purge the system for 5-10 minutes at a moderate flow rate. Gas should be bubbling through the KOH and then escaping from the flask into the atmosphere. Again, be sure that the fume hood is ventilating well.
11. Turn off the argon (or nitrogen) source, and allow the fume hood to continue to ventilate for 10-15 minutes to ensure that all traces of NO gas are removed from the atmosphere.
12. The solution of distilled water should now be saturated with NO. The concentration of NO produced by this saturation is dependent upon the temperature. At 0°C, the concentration is approximately 3.3mM, and at 20°C the concentration is approximately 1.91 mM.
13. Dilutions of known concentration can be prepared from this saturated solution. In preparing a dilution, **be careful not to unseal the vial**, for this exposes the solution to atmospheric oxygen.

Once the dilutions are prepared, it is a simple matter to calibrate the instrument.

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## **INSTRUMENT MAINTENANCE**

### **Storing the Sensor**

**STANDBY:** When not being used for a short period of time (such as overnight), the microsensor should remain attached to the microsensor cable and kept dry (not immersed in solution). Before the next experiment, immerse the sensor in the experimental solution (like, Krebs's Buffer). The background current will increase until reaching a stable value. Do not be alarmed if the background current becomes elevated. This is associated with the hydration of the sensor and will not negatively affect the sensor's performance.

**LONG-TERM:** If the microsensor will not be used for more than three or four days, then it may be stored dry by removing it from the microsensor cable and returning it to the case in which it was shipped, being very careful to avoid making contact with the sensor tip.

The NO microsensor is a maintenance-free consumable sensor. When its performance is no longer satisfactory, remove it from the microsensor cable and dispose of it, replacing it with a new one.

### **Cleaning the Sensor**

The sensor should be cleaned after each use by suspending the tip in distilled water for 20-30 minutes to dissolve salts and remove particles which may have accumulated on the membrane. If the sensor was used in a protein-rich solution, the tip should first be soaked in a protease solution for several minutes to remove protein build-up and then rinsed with distilled water. Enzymatic detergent (Enzol, WPI #7363-4) can also be used. The sensor can be sterilized chemically using an appropriate disinfectant (Cidex, WPI #7364). If necessary, gently dab the sensor with a Kimwipes® to remove residue.

**NOTE:** ALWAYS rinse with distilled water before storing the sensor dry.

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## ACCESSORIES

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**Table 1: Accessories**

<b>Part Number</b>	<b>Description</b>
7363-4	Enzol Enzymatic Detergent, 1 gallon
7364	Cidex Disinfecting Solution
91580	Microsensor Cable
47510	ProGuide Position/Holder with Base
47520	ProGuide Position/Holder
47530	ProGuide Plus Position/Holder with fine adjustment
47540	ProGuide Plus with Base
SNAP25	SNAP, 25mg vial
SNAP50	SNAP, 50mg vial
SNAP100	SNAP, 100mg vial

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## TROUBLESHOOTING

Issue	Possible Cause	Solution
Baseline current is below specified range.	The poise voltage (sensor setting) may be incorrectly set.	Set the poise voltage to 865mV. (For the TBR, choosing the NO sensor setting selects 865mV automatically.) Set the range at 10nA.
	The sensor may be nearing the end of its usable life.	Perform a 5-point calibration set using the standard. If the sensor responds linearly within the desired concentration range, it is still useable. See “Calibrating the Sensor” on page 6.
Unstable baseline	If the baseline hasn’t stabilized after 2 hours, the polarizing solution may be contaminated.	Prepare fresh polarizing solution. Use 0.1M CuCl <sub>2</sub> only.
	External electrical interferences may be the problem.	Identify and isolate electrical interferences.
Calibration dataset is not linear.	Uneven aliquots may have been used.	Check the pipetter calibration.
	The stock solutions have deteriorated.	Prepare fresh standard solution. See “Calibrating the Sensor” on page 6.
Sensitivity below range specified	Foreign materials have been adsorbed on the sensor’s surface.	If the foreign materials are proteins, use and enzymatic cleanser like Enzol (WPI #7363-4) to remove the contaminant.
	The sensor has reached end of its usable life.	Replace the sensor.

**NOTE:** If you have a problem/issue with your **NO Microsensor** that falls outside the definitions of this troubleshooting section, first perform the CuCl<sub>2</sub> Calibration Procedure (Method 2) exactly as describe on page 8 of this manual and contact the WPI Technical Support team at 941.371.1003 or technicalsupport@wpiinc.com.

## SPECIFICATIONS

The **NO Microsensors** conform to the following specifications:

	NOPF200	NOPF200-L10	NOPF100	NOP70-L	NOP3005	NOP3020	NOP30-L	NOP007	NOPNM
Outside Diameter (µm)	200	200	100	70	30	30	30	7	Conical tip:100
Available Length <sup>2</sup> (mm)	1-5 <sup>1</sup>	10	1-5 <sup>1</sup>	3	0.5	2	3	2	150µm
Response Time (sec.)	< 5	< 5	< 5	< 3	< 3	< 3	< 3	< 3	< 3
Lowest Detection Limit/Range (nM)	0.2	0.2	0.2	1	1	1	1	0.5	0.5
Nominal Sensitivity-New Sensor <sup>2</sup> (pA/nM)	≥20	≥50	≥10	≥10	≥1	≥1.5	≥1	≥1	≥0.5
Baseline Drift	none	none	none	none	none	none	none	none	none
Poise Voltage (mV)	865	865	865	865	865	865	865	865	865
Typical Quiescent Base-line Current, 25°C (pA)	2500	3500	2000	3500	1000	500	500	300	300
Acceptable Baseline Range (pA)	500-8000	500-8000	500-8000	2000-6000	150-3500	500-5000	20-6500	200-1500	50-100
Polarization Time (hrs)	2+	8+	2+	2+	1+	1+	1+	1+	1+

<sup>1</sup>Sensor length varies in 1mm increments (for example, 1mm, 2mm, 3mm...).

<sup>2</sup>Sensor length is proportional to sensitivity.

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## APPENDIX A: PREPARING STOCK SOLUTION

### 100 $\mu$ M Standard SNAP Solution

SNAP is a green crystalline compound that is sold in 25mg, 50mg and 100mg vials (WPI # **SNAP25**, **SNAP50**, **SNAP100**). Both the crystalline form and the liquid solution of SNAP are photo-sensitive and tend to degrade over time. Wrap the vial of SNAP compound in aluminum foil and store it in the freezer to slow its degradation. Similarly, store the bottle of SNAP solution in an amber bottle or wrap it with aluminum foil and store it in the refrigerator.

**NOTE:** The decomposition of SNAP at low temperature, in the dark and in the absence of trace metal ions proceeds slowly because of the EDTA (a chelating reagent).

WPI technicians recommend making fresh standard SNAP solution daily to ensure accurate calibration of NO sensors. Concentration of SNAP decreases to 5-10% of its nominal value after approximately 4-5 hours.

To make a 100 $\mu$ M solution of SNAP:

1. Accurately weigh out 5.0mg EDTA (a preservative) and place it in a clean, dry bottle that will hold at least 250mL.
2. Use a clean, dry 250mL volumetric flask to accurately measure 250mL of HPLC pure water (HPLC grade, Sigma).

**TIP:** If your research demands an oxygen-free sample, you can de-oxygenate this solution by purging it with pure nitrogen or argon gas for 15 minutes.

3. Pour the water into the bottle with EDTA. Replace the cap and shake it for a few seconds to dissolve the EDTA. It dissolves rapidly.
4. Accurately weight out 5.6mg of crushed, crystalline SNAP.

**TIP:** Crush any clumps of SNAP powder with a clean instrument like a glass stirring rod, a popsicle stick or a tooth pick. If you prefer, place the 5.6mg SNAP on a small piece of filter paper, fold the paper in half and rub it gently between your fingers to break up any clumps. Be careful not to spill any of the compound.

5. Add the 5.6mg SNAP to the EDTA solution. Verify that none of the green SNAP compound is left on your filter paper or measuring tray. Replace the cap and shake it for a few seconds until the green flecks dissolve into solution.
6. Store this standard solution in an amber bottle, if available, or alternatively, wrap aluminum foil around the bottle to limit light intrusion. This solution should be refrigerated.

The concentration of SNAP (f.w.= 220.3) in the stock solution is calculated as follows:

$$[C] = [A \cdot W / (M \cdot V)] 1000$$

[C] = concentration of SNAP ( $\mu\text{M}$ )

A = purity of SNAP

W = weight of SNAP (mg)

M = formula weight of SNAP (220.3g/mol)

V = volume of the solution (L)

If SNAP purity is 98.5%, the concentration of standard SNAP stock solution describe above is:

$$[C] = [0.985 \times 5.6\text{mg} / (220.3\text{g/mol} \times 0.25\text{L})] \times 1000 = 100.1\mu\text{M}$$

**NOTE:** The purity of SNAP used is extremely important to ensure an accurate calibration. We recommend the use of high grade SNAP with minimal purity of 98% or better.

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## APPENDIX B: CONSTRUCTING A CALIBRATION CURVE

Because NO sensors can be calibrated in a linear fashion, the magnitude of every signal should almost double as the volume of SNAP solution added is doubled in the course of the calibration. The response should be linear from 10 to 100nM.

Use the recorded data to construct a calibration curve by plotting the signal output (for example, in pA) against the concentration of SNAP added at that time. Note that every addition of SNAP solution corresponds with a particular NO concentration. The sensitivity of the NO probe can be established from the gradient or slope of the response curve. After the sensitivity of the NO probe is established, software (like Data-Trax) can be programmed to display data in either concentration directly (nM or mM) or redox current (pA or nA).

### Predicting the Level of Detectable NO CuCl<sub>2</sub>

Experiments have shown that SNAP is decomposed instantaneously under the following set of experimental conditions:

- Temperature 25°C
- Catalyst solution 0.1M CuCl<sub>2</sub>
- SNAP (WPI, 98% purity) - Fresh standard SNAP solution
- CuCl<sub>2</sub> is at equilibrium with ambient air (aerobic conditions)

SNAP (RSNO) decomposes to NO and a disulfide byproduct according to the following equation:



Theoretically, the concentration of generated NO should be equal to the final concentration of SNAP in the copper chloride solution in the calibration vial if the decomposition goes to completion and if the generated NO is detected quickly before it is oxidized to nitrite and nitrate. However, it is expected that the level of detectable NO will be below the theoretical value, because the copper chloride solution is at equilibrium with ambient air, and consequently a portion of the generated NO would have been immediately oxidized to nitrite and nitrate before it was measured by the NO sensor. In addition, it is possible that decomposition of SNAP does not go to completion even in the presence of a catalyst. Results on the kinetics of SNAP decomposition in the presence of a catalyst in an anaerobic environment are published elsewhere (Zhang et al., "Novel Calibration Method for Nitric Oxide Microsensors by Stoichiometrical Generation of Nitric Oxide from SNAP", *Electroanalysis*, 2000, 12: 6, 425-428).

**NOTE:** When using the CuCl<sub>2</sub> method, experimental data indicates a conversion efficiency of SNAP to NO of approximately 0.6 (60%). This result is only applicable for calibration of a NO sensor in a solution, which is at equilibrium with ambient air and at the experimental conditions described above. Hence for each mole of SNAP, 0.6 mole of NO is liberated under the proposed set of parameters. It is assumed the

other 40% of SNAP is either not decomposed or a proportion that is decomposed to NO is subsequently oxidized immediately before it is detected by the NO sensor.

## Example for Creating a Calibration Curve

The following example walks through the CuCl<sub>2</sub> calibration procedure.

1. Prepare the solutions.
2. Calculate the concentration of NO in the SNAP solution (molarity).

$$M = m/V$$

Where M is the concentration in molar, m = mass of the substance in moles,  
V= volume of solution in liters

$$M = [(5.6 \times 10^{-3} \text{g} * 98.5\%) / 220.3 \text{g/mol}] / 0.25 \text{L} = 100.1 \mu\text{M}$$

3. Set up the equipment. Place 20mL of Solution #1 (CuCl or CuCl<sub>2</sub>) in a vial on the magnetic stirring plate and set up the NO probe. Allow the background current to stabilize before recording.
4. Calculate the total volume in the vial after each addition and record the number. The initial value is 20mL (0.02L). For a five point calibration, the concentrations to be added are 2μL (2x10<sup>-6</sup>L), 4μL (4x10<sup>-6</sup>L), 8μL (8x10<sup>-6</sup>L), 16μL (16x10<sup>-6</sup>L) and 32μL (32x10<sup>-6</sup>L). The successive total values after each addition are 0.020002L, 0.020004L, 0.020008L, 0.020016L and 0.020032L.
5. Calculate the concentration of SNAP in each aliquot to be added to the 20mL of Solution #1. Calculate the final concentrations for each addition using the formula for dilutions.

$$M_i V_i = M_f V_f \text{ where}$$

M<sub>i</sub> = initial molarity

M<sub>f</sub> = final molarity

V<sub>i</sub> = initial volume

V<sub>f</sub> = final volume

$$[100.1 \mu\text{M} * 2 \mu\text{L}] / 0.020002 \text{L} = 10.00 \text{nM}$$

$$[100.1 \mu\text{M} * 4 \mu\text{L}] / 0.020006 \text{L} = 20.01 \text{nM}$$

$$[100.1 \mu\text{M} * 8 \mu\text{L}] / 0.020014 \text{L} = 40.01 \text{nM}$$

$$[100.1 \mu\text{M} * 16 \mu\text{L}] / 0.020030 \text{L} = 79.96 \text{nM}$$

$$[100.1 \mu\text{M} * 32 \mu\text{L}] / 0.020062 \text{L} = 159.66 \text{nM}$$

6. Calculate the effective concentration of NO in the solution after each addition is made. When using the CuCl<sub>2</sub> calibration method, the yield of NO gas is approximately 60% of the concentration of SNAP.

$$[\text{SNAP}] * 0.6 = [\text{NO}]$$

$$10.00 \text{nM} [\text{SNAP}] * 0.6 = 6.00 \text{nM} [\text{NO}]$$

$$20.01 \text{nM} [\text{SNAP}] * 0.6 = 12.00 \text{nM} [\text{NO}]$$

$$40.01 \text{nM} [\text{SNAP}] * 0.6 = 24.00 \text{nM} [\text{NO}]$$

$$79.96 \text{nM} [\text{SNAP}] * 0.6 = 47.97 \text{nM} [\text{NO}]$$

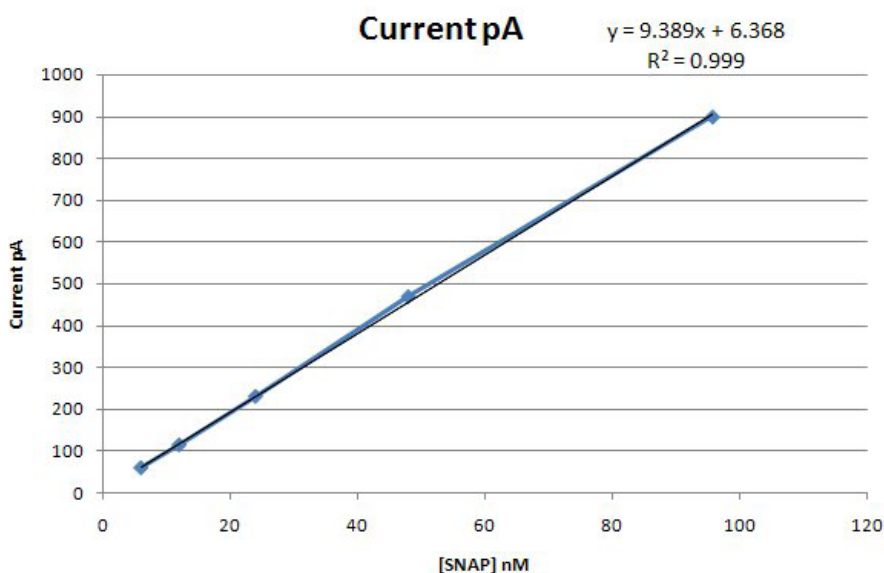
$$159.66 \text{nM} [\text{SNAP}] * 0.6 = 95.79 \text{nM} [\text{NO}]$$

7. Record your calculated values in a table similar to the one in step 8.
8. Add the test aliquots and measure the current output. The SNAP reacts with

the  $\text{CuCl}_2$  producing NO gas. When the sensor detects NO gas, it generates an output current that is measurable, and the results can then be graphed. To the vial containing 20mL of solution #1, sequentially add the five aliquots of standard SNAP solution (Solution #2), recording the current measurements after each addition in the last column of the table below. For this example the values are recorded from a new ISO-NOPF100 sensor.

Calculated Values:				Measured Values:
Aliquot	Total volume	[SNAP]	[NO]	Current Output
2 $\mu\text{L}$	0.020002L	10.00nM	6.00nM	60pA
4 $\mu\text{L}$	0.020006L	20.01 nM	12.00nM	115pA
8 $\mu\text{L}$	0.020014L	40.01 nM	24.00nM	231 pA
16 $\mu\text{L}$	0.020030L	79.96nM	47.97nM	470pA
32 $\mu\text{L}$	0.020062L	159.66nM	95.79nM	900pA

- Construct a standard calibration curve using the recorded data. Using a spreadsheet with graphing capability like Microsoft<sup>®</sup> Excel, it is possible to generate a linear regression analysis that will display the equation and the  $R^2$  coefficient. To do this in Excel, enter the data and generate a "scatter plot" graph. Then, select the line and right click. Choose **Add Trendline**. The **Format Trendline** dialog box appears. On the **Trendline Options** tab, select **Linear**, **Display equation on chart** and **Display R-value on chart**. (See Fig. 4.)



**Fig. 4** – Scatter plot of data from example

The data from the calibration curve indicates that this procedure yields an excellent linear calibration of NO probes. The accuracy of calibration is approximately  $\pm 10\%$  from mean. The source of error arises most probably from gravimetric measurement of the standard reagent, SNAP. In addition, purity of SNAP (as well as partial oxidation of generated NO in the calibration solution) could contribute to this error. Such a deviation may not be so important when NO is quantified in biological systems, because most often the ability to measure changes in the basal concentration of NO is more significant than measurement of the absolute level of NO.

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